

## Tumorigenesis and Neoplastic Progression

# Fluorescence *in Situ* Hybridization Markers for Prediction of Cervical Lymph Node Metastases

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**The presence of lymph node metastases is associated with poor prognosis in early stage cervical cancer. As of yet, no molecular markers predicting lymph node metastases have been identified. We examined single genetic markers and a composite marker, comprised of three fluorescence *in situ* hybridization (FISH) probes targeting the genes *LAMP3*, *PROX1*, and *PRKAA1*, in pretreatment cervical biopsies from 16 lymph node positive cases and 15 lymph node negative controls from women with stage IB and IIA cervical cancer. In addition, we determined clonal patterns by including *CCND1* to compare the clonal constitution of primary tumors and associated lymph node metastases. The composite FISH marker allowed for classification of patients into those with and without lymph node metastases with a sensitivity and specificity of 75% and 87%, respectively ( $P = 0.001$ ). The positive predictive value and negative predictive value were 86% and 76%, respectively. Clonal patterns varied among the tumors. In many cases, changes between the primary tumor and lymph node metastases in the most common clones may indicate that certain clones have a growth advantage for establishing metastases in lymph nodes. We conclude that the composite FISH marker may be useful for determining risk for subsequent develop-**

**ment of lymph node metastases in patients with cervical cancer. (Am J Pathol 2009; 175:2637–2645; DOI: 10.2353/ajpath.2009.090289)**

Cervical cancer is globally the second most common tumor in women, with 80% of the cases occurring in developing countries.<sup>1–3</sup> Overall survival is generally high among patients with early stages of cervical cancer; however, the presence of lymph node metastases significantly reduces survival by a factor of four in stage Ib1 disease, therefore constituting the strongest prognostic factor for survival.<sup>4–6</sup> Molecular markers as a means to detect lymph node metastases may therefore have therapeutic implications.

Genomic alterations in cervical cancer have been reported in many studies with consistent gains and amplifications found on chromosomes 1q, 3q, and 5p.<sup>7–11</sup> Potential genes of interest in these regions of amplification include *LAMP3*, *PROX1*, and *PRKAA1*. *LAMP3* resides on a region of high importance for cervical carcinogenesis, with high expression found to be associated with an enhanced metastatic potential in cervical cancer.<sup>12</sup> The homeobox gene, *PROX1*, is a lymphatic endothelium specific marker involved in the developmental regulation of the lymphatic system.<sup>13–15</sup> *PRKAA1*, a cellular metabolic stress regulator, may assist tumor cell growth under stress and is a potential cervical carcinogenesis marker.<sup>16</sup> In addition to these genes, we included *CCND1*, a genetic marker of cellular proliferation. *CCND1* is located on chromosome 11q13 and altered expression of this gene has been observed in many cancers. *CCND1* overexpression is also associated with lymph node metastases in oral cancer.<sup>17</sup>

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We analyzed genomic copy numbers of *LAMP3*, *PROX1*, *PRKAA1*, and *CCND1* by fluorescence *in situ* hybridization (FISH) in pretreatment cervical biopsies from lymph node positive (cases) and lymph node negative (controls) stage IB and IIA patients with cervical cancer to explore their role as potential predicting factors for lymph node metastasis. In addition, we also analyzed the four markers in lymph node metastases from all cases with the objective to identify clonal patterns indicative of lymphatic spread.

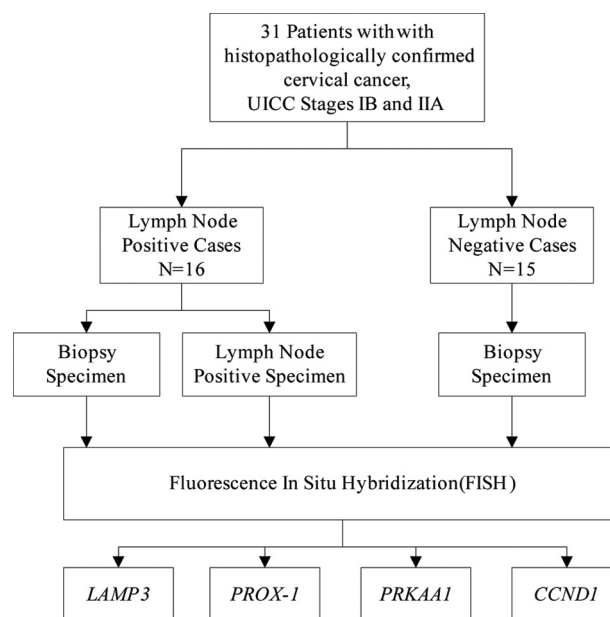
## Materials and Methods

Pretreatment formalin-fixed, paraffin-embedded biopsy specimens were obtained from 31 patients with cervical cancer at stages IB and IIA according to the International Federation of Gynecology and Obstetrics staging system, treated at the Department of Gynaecologic Oncology, Radiumhemmet (Stockholm, Sweden), from January 1994 to December 1997. Clinical information, including age, International Federation of Gynecology and Obstetrics stage, histology, tumor size, grading, lymph node metastasis, treatment modality, and follow-up status was retrieved from medical records. Radical hysterectomy and pelvic lymphadenectomy was performed in all patients. Preoperative intracavitary brachytherapy (remote after-loading technique with caesium) was given to 25 patients in the form of two uterovaginal insertions with a 3-week interval followed by surgery, 4 weeks after the second insertion. Of the 31 patients, 16 had histologically verified lymph node metastases (cases) and 15 were lymph node negative (controls). From all patients that presented during the observation time, we selected 31 patients that could be matched according to stage, grade, size, and lymph vascular invasion. In addition to the biopsy specimens already collected, we obtained samples from the positive lymph nodes. All histological samples were reviewed by a pathologist who was blinded to clinical outcome to confirm representative tumor specimens. The study was approved by the research ethical review board (Dnr: 01-269) at Karolinska Institutet. Pictorial representation of the experimental design is shown in Figure 1.

FISH was performed on 47 samples. Two 4- $\mu$ m sections were cut before and after the FISH sections and H&E stained, to confirm tumor representation. Images for a primary tumor and its corresponding lymph node metastasis of the H&E stained sections, FISH on adjacent paraffin section, and FISH on cytopspins prepared from adjacent paraffin sections are shown in Figure 2, A–F.

## Sample Preparation and FISH

FISH was performed by using a centromere-specific probe for chromosome 7 (CEP7; Abbott Molecular, Inc; Des Plaines, IL), and bacterial artificial chromosome (BAC) contigs for the following four probes: *LAMP3* probe at chromosome band 3q26; *PROX1* probe at chromosome band 1q41; *PRKAA1* probe at chromosome band 5p19; and *CCND1* probe at chromosome 11q13. Images

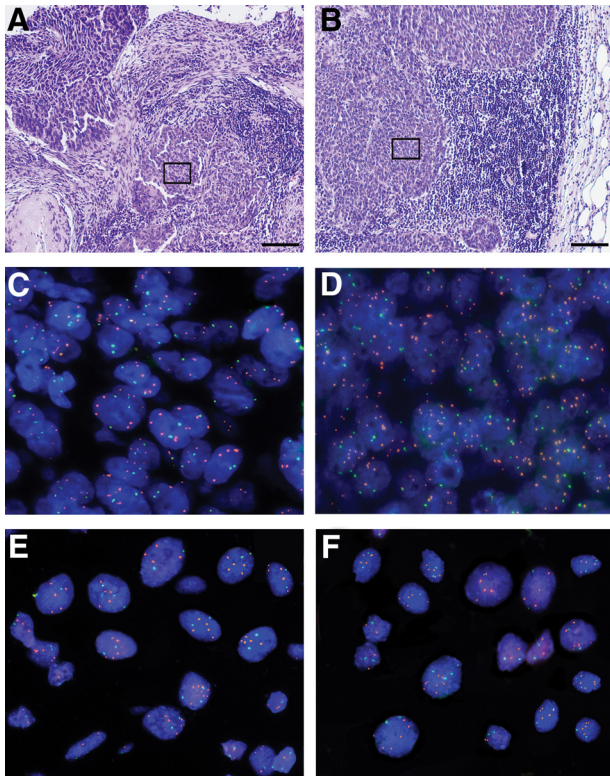


**Figure 1.** Pictorial representation of the experimental design.

of the individual FISH signals used are shown in Figure 3, A–F. The BAC contig of each probe comprised of four to five overlapping BAC sequences specific to the region of interest for each probe. The CEP7 probe (Abbott Molecular, Inc) was labeled in Spectrum Aqua. The BAC clone contigs were labeled by nick-translation with Spectrum Orange-dUTP (Abbott Molecular, Inc) for *LAMP3*, with Rhodamine Green-dUTP (Life Technologies; Carlsbad, CA) for *PROX1*, with Spectrum Orange-dUTP (Abbott Molecular, Inc) for *PRKAA1*, and with Rhodamine Green-dUTP (Life Technologies) for *CCND1*. The FISH markers were combined into two probe panels, with the first probe combination consisting of *LAMP3*, *PROX1*, and CEP7 (Abbott Molecular, Inc). The second probe combination contained *PRKAA1* and *CCND1*. The two probe panels were subsequently hybridized to the samples, resulting in counts for all five FISH probes within the same nuclei.

For accurate signal number per nucleus enumeration, single nuclei preparations were prepared from 6- $\mu$ m formalin-fixed, paraffin-embedded sections. The tissue was deparaffinized by using xylene (three times for 10 minutes) and rehydrated by using an ethanol series before centrifugation at 14,000 rpm. Excess 50% ethanol was removed before undergoing 20-minute incubation in sterile water at room temperature. The cells were then digested by adding 0.1% Protease (Type XXIV, Sigma, St. Louis, MO, USA) in 1 X PBS at 45°C for 45 to 60 minutes. Once an optimal single cell suspension was achieved, the reaction was stopped with 1 X PBS. The cells were then deposited onto slides by centrifugation, fixed in ethanol for 10 minutes, air dried, and stored at 4°C.

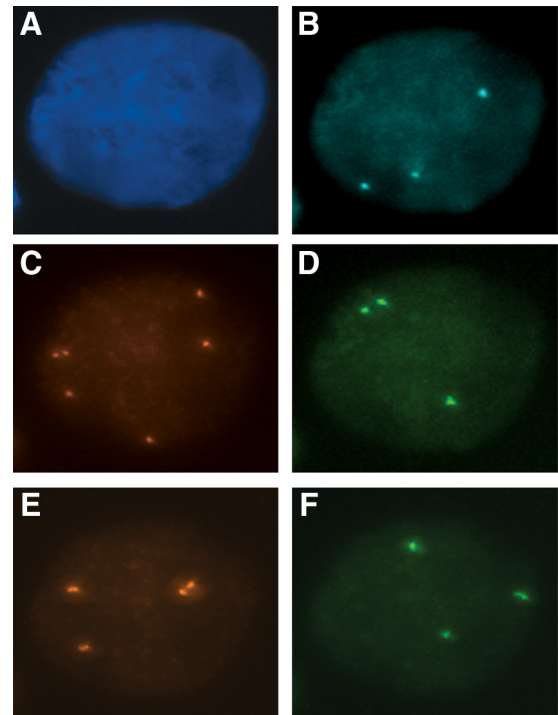
For hybridization, slides were pretreated with 0.05% pepsin for 20 to 25 minutes before undergoing fixation in 1% formaldehyde for 10 minutes followed by ethanol dehydration. After air drying, the slides were denatured in a 70% formamide/2 X saline sodium citrate (SSC) solution for 2 minutes. The slides were then put through an ice



**Figure 2.** Histological and FISH images of primary tumor C18 (A, C, and E) and its corresponding lymph node metastasis C19 (B, D, and F). A and B: Images of H&E stained sections showing areas representative for cervical cancer and cervical cancer lymph node metastasis, respectively. The length of the black bar in the bottom right corner is equivalent to 100  $\mu$ m. The black frames indicate the tissue area shown in a larger magnification in the FISH images below. C and D: FISH probes *PROX1* (green signals) and *LAMP3* (orange signals) hybridized on sections corresponding to the H&E sections shown in A and B. The main signal numbers observed per nucleus were two signals for *PROX1* and four to five signals for *LAMP3*. E and F: FISH probes *PROX1* (green signals) and *LAMP3* (orange signals) hybridized on cytopsin preparations prepared from sections corresponding to the H&E sections shown in A and B. The main signal numbers observed per nucleus were two signals for *PROX1* and four to five signals for *LAMP3*. Note the improved signal countability in the cytopsin compared with the sections (C and D). Most of the samples of this study had histological sections with highly overlapping nuclei, which made accurate enumeration impossible. We therefore disintegrated the sections and prepared monolayer cytopsin from the resulting single cell suspensions.

cold dehydrating ethanol series (70%, 90%, and 100%) and air dried. All probes were denatured for 5 minutes at 80°C followed by preannealing at 37°C for 2 hours except for CEP7 (Abbott Molecular, Inc), which requires no preannealing. Preannealed probes were mixed with the denatured CEP7 probe (Abbott Molecular, Inc) before being added to the denatured slides. The slides were then coverslipped and sealed with rubber cement before being placed in a humidified chamber for overnight hybridization at 37°C. After hybridization, slides were washed in 2  $\times$  SSC (three times for 3 minutes each time) followed by a dehydrating ethanol series (three times for 3 minutes each time). The slides were counterstained with a 4,6-diamidino-2-phenylindole (antifade) solution and mounted with a coverslip.

Hybridized FISH slides were viewed by using the Leica DM-RXA fluorescence microscope (Leica; Wetzlar, Germany) equipped with custom optical filters and a 40 $\times$  objective. The Leica CW 4000 FISH software was used to



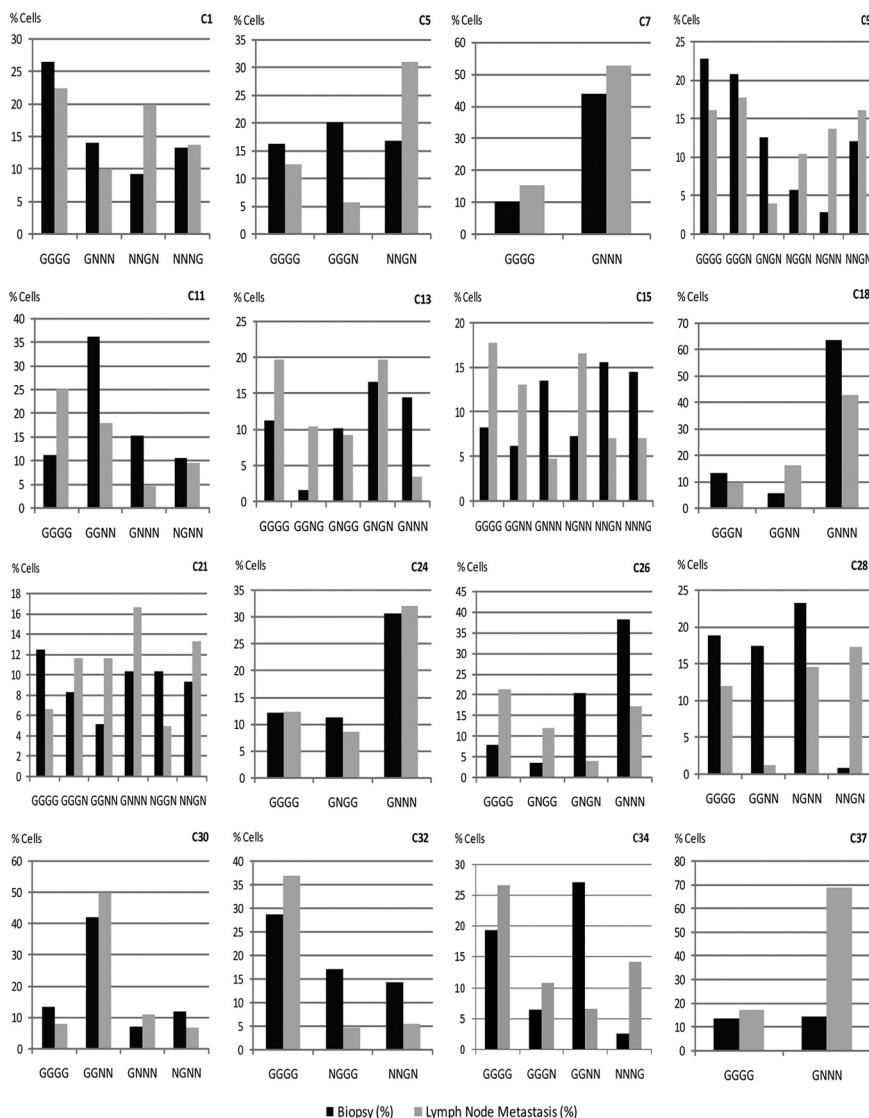
**Figure 3.** FISH images from a cytopsin prepared from one of the primary biopsies (A) 4',6'-diamidino-2-phenylindole hydrochloride stained cells, (B) CEP7 (Abbott Molecular, Inc) (Aqua), (C) *LAMP3* signals (orange), (D) *PROX1* signals (Green), (E) *PRKAA1* signals (orange), and (F) *CCND1* signals (Green).

acquire multifocal images for each filter. Fifteen to 25 images were taken in areas of optimal cell density with minimal cellular clumps and overlapping cells. Stage relocation was used to recapture the same cells hybridized with the second probe combination. Once imaging and relocation were completed for the first probe set, the probes were washed off with 50% formamide/SSC at 80°C for 2 to 3 minutes before undergoing ethanol dehydration. The air dried slides were then denatured in 70% formamide/2  $\times$  SSC at 80°C for 1 minute before being dehydrated in ice cold ethanol series. Once the slides air dried, the second probe combination (probes were denatured/preannealed according to the above protocol) was applied to the slide for overnight hybridization at 37°C. Probes were detected according to the protocol of the first probe combination. Images were acquired at the exact same regions as the first probe panel. Each hybridization set contained a lymphocyte control from a normal human donor.

### FISH Analyses

FISH analyses were performed on all 47 samples with approximately 250 interphase cells counted in each case. The four gene probes and the CEP7 probe (Abbott Molecular, Inc) were scored within each of the 250 cells. The CEP7 probe (Abbott Molecular, Inc) was used to estimate the ploidy of the cell, since it was previously shown that chromosome 7 is the least involved chromosome in cervical carcinogenesis.<sup>7,10</sup> Nuclei that could not be evaluated due to various rea-





**Figure 4.** Clonal patterns observed in 16 primary tumor biopsies and their corresponding lymph node metastases. Each graph represents a comparison of signal patterns between the primary tumor (black bars) and the lymph node metastasis (gray bars) of one patient with the primary biopsy case number depicted in the **right upper corner**. For this analysis, each nucleus was enumerated for the control probe (CEP7 [Abbott Molecular, Inc]) and gene probe (*LAMP3*, *PROX1*, *PRKAA1*, and *CCND1*) signals. A gain (G) of a certain gene probe within a nucleus was defined by a signal ratio >1 of the respective gene probe divided by the control probe CEP7 (Abbott Molecular, Inc), while a not gained (N) signal number was reflected by a ratio of the gene probe divided by CEP7 (Abbott Molecular, Inc) of  $\leq 1$ . Signal patterns observed in >10% of the cell population are displayed on the x axis of the graphs according to gained signals (G) or not gained (N) signals for the respective gene probes, with the first letter in the pattern corresponding to *LAMP3*, the second letter to *PROX1*, the third to *PRKAA1*, and the fourth to *CCND1*. The y axis presents the percentage of nuclei observed with the respective pattern. Signals pattern examples: GGGG = Gained signals seen for all four markers; NNGN = No gains seen in *LAMP3*, *PROX1*, and *CCND1*. Gain is seen in *PRKAA1*.

sons, including overlaps and insufficient hybridization, were excluded. Scoring of FISH markers was completed without prior knowledge of clinical outcome or histopathological evaluation.

FISH markers (*LAMP3*, *PROX1*, *PRKAA1*, and *CCND1*) were compared against the corresponding CEP7 probe (Abbott Molecular, Inc) in each cell and a ratio was calculated (FISH marker divided by CEP7 [Abbott Molecular, Inc]). A ratio per cell of greater than one was considered a *gained* signal. A ratio per cell of more than two was considered an *amplified* signal. Calculations were made according to the percentage of cells with gained or amplified signals within each case. The FISH composite marker status was based on the percentage of cells with amplified signals, relative to optimized thresholds established for each single FISH marker. For single FISH markers, cases with scores above the optimized threshold were considered a positive test. For the FISH composite marker, cases exhibiting a positive test for all three markers (*LAMP3*,

*PROX1*, and *PRKAA1*) were considered a FISH positive test.

### Statistical Analyses and Clonal Pattern Observations

Statistical analyses were performed with SAS version 9.1 (SAS Institute; Cary, NC), using the Mantel-Haenszel  $\chi^2$  test to test patient characteristics with all FISH scores. The sensitivity (identifying specimens with lymph node metastasis) was compared with the specificity (identifying specimens without lymph node metastasis) by using the receiver operator characteristic (ROC) plot for the FISH composite marker. Sensitivities and specificities were calculated by independently varying the thresholds for all three probe signals over a wide range. Only the highest sensitivity value for each value of the specificity is plotted, with curves coming closest to 100% sensitivity and specificity (the ideal point, upper left corner of the graph) being optimal. The

distance from ideal plot depicts the distance from the ideal point on the ROC plot as a function of threshold, with minimums identifying optimal threshold values, as previously described by Heselmeyer-Haddad et al.<sup>18</sup>

Clonal patterns were described as the association between four single FISH markers as evaluated in a single cell basis. The single FISH markers used in determining clonal patterns were compared against the CEP7 (Abbott Molecular, Inc) probe in each cell with ratios calculated (FISH marker divided by CEP7 [Abbott Molecular, Inc]). Gained (G) signals correspond to ratios greater than one and not gained (N) signals correspond to ratios of one or less than one. Clonal patterns, as seen in Figure 4, depict the percentages of cells with the observed gained (G) or not gained (N) signal combination for the four FISH markers (*LAMP3*, *PROX1*, *PRKAA1*, and *CCND1*). Patterns are listed by markers, with the first letter corresponding to *LAMP3*, then *PROX1*, *PRKAA1*, and *CCND1*. Example: GGGG = Gained signals for all four markers; NNGN = no gains for *LAMP3*, *PROX1*, and *CCND1*, but gain for *PRKAA1*.

## Results

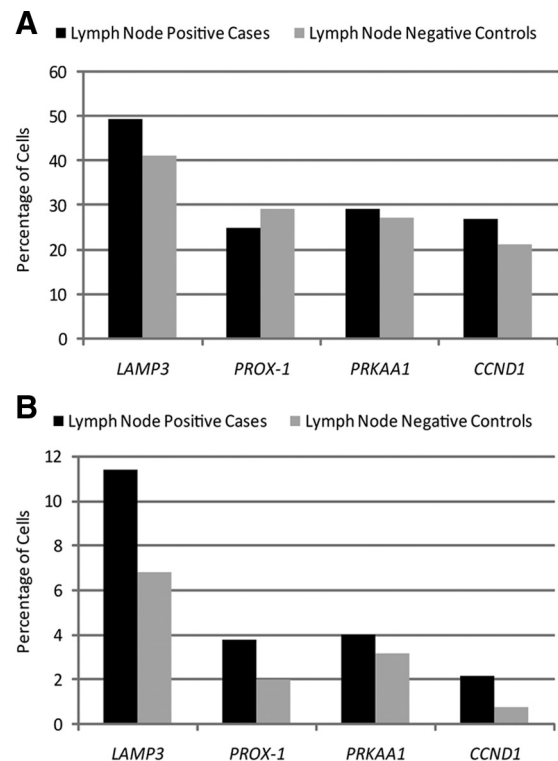
Clinical characteristics are presented in Table 1. Almost half (44%) of the lymph node positive patients died, while all of the lymph node negative patients remained alive during the 10-year follow-up period. Of the seven patients who died, lymph vascular invasion was observed in two patients.

**Table 1.** Baseline Characteristics of Patients with Primary Cervical Cancer with (Cases) and without (Controls) Pelvic Lymph Node Metastases

Characteristics	Cases, n = 16	Controls, n = 15
Age at diagnosis, yr		
Median	41.5	47
Range	29–66	34–69
Stage*		
IB	12	13
IIA	4	2
Tumor size		
≤4 cm	8	7
>4 cm	8	8
Histology		
Squamous cell carcinoma	10	10
Adenocarcinoma	2	2
Adenosquamous carcinoma	4	3
Histopathologic grade†		
2	6	6
3	9	8
Lymphovascular invasion		
Present	5	2
Absent	11	13
Treatment		
Preoperative brachytherapy	14	11
Radical hysterectomy and pelvic lymphadenectomy	16	15
Survival		
Alive	9	15
Dead	7	0

\*Tumor stage according to the International Federation of Gynecology and Obstetrics.

†Tumor differentiation grade according to the World Health Organization international histologic classification of tumors.



**Figure 5.** Comparison between lymph node positive (black bars) and lymph node negative cases (gray bars) for the median percentage of cells with gained (gene probe divided by CEP7 [Abbott Molecular, Inc] signal ratio >1) signals for the respective gene probes (A) and the median percentage of cells with amplified (gene probe divided by CEP7 [Abbott Molecular, Inc] signal ratio >2) signals for the respective gene probes (B).

FISH analyses were successfully performed on all 31 pretreatment cervical biopsies and 16 corresponding lymph node metastases. FISH results were analyzed in comparison with patient characteristics within the lymph node positive and negative cases, but none of these comparisons revealed differences that were statistically significant. Copy number gains were seen in all 31 biopsy specimens for all four markers. *LAMP3* was the only marker where amplified cells were observed in all cases and controls, with an average of 17% of cells containing an amplified signal. The remaining three markers (*PROX1*, *PRKAA1*, and *CCND1*) each had no amplified signals in several cases. FISH marker comparisons of gained and amplified signals in lymph node positive and negative cases are depicted in Figure 5, A and B. When comparing amplified signals in lymph node positive and negative cases, we observed a larger percentage of amplified nuclei in samples with lymph node metastases, especially with the *LAMP3* marker.

## FISH Marker Combinations Predicts Lymph Node Metastases

A combined FISH marker consisting of probes for *LAMP3*, *PROX1*, and *PRKAA1* provided a significant ( $P = 0.001$ ) predictor for lymph node metastases (Table 2). The optimized threshold used in the composite marker was according to percentages of cells with amplified

**Table 2.** *P*-values to Distinguish between Lymph Node Positive Cases and Lymph Node Negative Controls by Combining Single FISH Markers into a Composite Marker in Pretreatment Cervical Biopsies

Marker	Optimized thresholds of amplified nuclei, %	Lymph node positive cases,* <i>n</i> = 16	Lymph node negative controls,* <i>n</i> = 15	<i>P</i> <sup>†</sup>
<i>LAMP3</i>	≥6.8	12	8	0.215
	<6.8	4	7	
<i>PROX1</i>	≥2.0	12	9	0.379
	<2.0	4	6	
<i>PRKAA1</i>	≥2.0	14	9	0.085
	<2.0	2	6	
<i>CCND1</i>	≥1.3	11	5	0.052
	<1.3	5	10	
Composite	Positive test	12	2	0.001
Marker <sup>‡</sup>	Negative test	4	13	

\*Cases with scores above optimized threshold for each single marker were considered a positive test.

<sup>†</sup>Mantel-Haenszel  $\chi^2$  test.

<sup>‡</sup>Composite marker includes *LAMP3*, *PROX1*, and *PRKAA1*, with each case requiring a positive test for all three markers to be considered a composite marker positive test.

signals for each individual marker (*LAMP3*: ≥6.8; *PROX1*: ≥2.0; *PRKAA1*: ≥2.0). *CCND1*, as a single marker, provided borderline significance ( $P = 0.052$ ), with most positive values having low percentages near zero. The specificity, sensitivity, positive predictive value, and negative predictive value of the composite FISH probes are shown in Table 3. These results indicate that 75% of lymph node positive patients were correctly identified with a positive test, while 87% of lymph node negative patients were correctly identified with a negative test. The positive predictive value represents the proportion of test positive patients (86%) who actually had the disease, while the negative predictive value at 76% represented the percentage of test negative patients who were correctly identified with a negative test result. The ROC curve illustrates the relationship between sensitivity and specificity of the FISH composite marker (Figure 6A). Figure 6B depicts the distance from ideal curves of all three single FISH markers used to determine the composite FISH marker and supports the selected threshold values (minimum in each curve). The composite FISH markers results were not related to tumor histology.

### Clonal Patterns

Examination of clonal patterns in tumor biopsies and the corresponding lymph node metastases provided evidence that in certain cases the proportions of different clones can vary between the primary tumor and the metastatic lymph node (Figure 4). Several cases (C7, C24, C30, C32, C37) showed similar clones and similar quan-

titative distribution between primary tumor and lymph node metastasis, indicating that the major clone(s) of the primary tumor were also the most successful clone(s) to establish lymph node metastasis. However, in many cases, a change in the frequency of specific clones was observed between the primary tumors and their associated lymph node metastases indicating that pre-existing, but minor clones in the primary tumors had a growth advantage in the lymph nodes. For example, in cases C1, C5, and C28, the major clonal change observed was the considerable increase of NNGN (gained signal seen in *PRKAA1* only, see *Materials and Methods*) in the lymph node metastases. Although similarities in clonal patterns of certain cases were observed, these resemblances were not related to the patients' clinical characteristics.

### Discussion

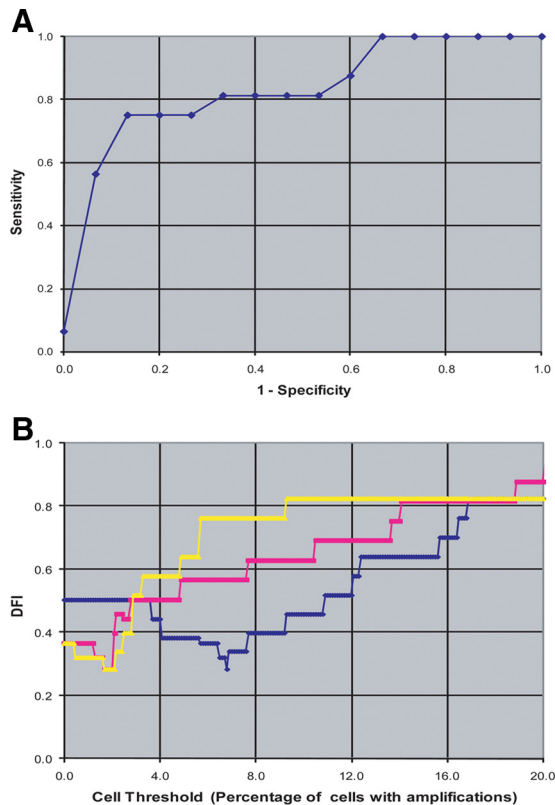
For cervical cancer, we found that the enumeration of genomic copy numbers of the composite marker consisting of *LAMP3*, *PROX1*, and *PRKAA1* allowed statistically significant prediction of lymph node metastases. We also observed that clonal patterns may differ between a primary tumor and its metastasis, indicating that certain clones may have a growth advantage in the lymph node environment.

To our knowledge, no other study has performed FISH analyses for the prediction of lymph node metastases in cervical cancer. Studies attempting to predict lymph node metastases have used techniques including immu-

**Table 3.** Positive Predictive Value, Negative Predictive Value, and Sensitivity and Specificity Tests for Composite FISH Markers (*LAMP3*, *PROX1*, and *PRKAA1*)

	Lymph node positive cases, <i>N</i>	Lymph node negative controls, <i>N</i>		
Positive test	12	2	14	→
Negative test	4	13	17	→
	16	15		
	↓	↓		
	Sensitivity = 0.75	Specificity = 0.87		

PPV, positive predictive value; NPV, negative predictive value.



**Figure 6.** **A:** ROC plot depicting specificity vs 1– sensitivity curves for the composite FISH marker **(B)** distance from ideal curves for the three individual markers (*LAMP3* in blue, *PROX1* in pink, *PRKAA1* in yellow) used in the FISH composite marker (see *Materials and Methods*).

nohistochemistry, RT-PCR, and gene expression profiling. Immunohistochemistry is a cost-effective technique, but diverse epitopes (Cox-2, p16, CXCR4, CCR7, D2-40, etc) and controversial results were reported for the prediction of lymph node metastases in cervical cancer.<sup>19–24</sup> Gene expression profiling studies also found divergent results with a study by Biewenga et al,<sup>25</sup> which was not able to predict lymph node metastases in early stage cervical cancer, while a study by Kim et al<sup>26</sup> did. Biewenga et al<sup>25</sup> found five genes showing differential expression between patients with and without lymph node metastases. The prediction of lymph node positivity will assist in developing adequate treatment options for patients since histologically undetectable micrometastases in the lymphatic system may account for cervical cancer recurrence.<sup>27–29</sup> Histological examinations are not infallible since examinations only comprise a small portion of lymph nodes, and estimates indicate that a pathologist has a 1% chance of detecting a micrometastatic focus within a three-tumor-cell diameter.<sup>28,30</sup>

Gene amplification is a common mechanism for oncogene activation in tumorigenesis and gains on chromosomes 1q, 3q, and 5p are frequently observed in cervical cancer.<sup>7–11</sup> In our study, a gain of chromosome 3q was seen in all samples, regardless of histology, which confirms the dominant role of this chromosome in the development of cervical cancer.<sup>7–11,18,31</sup> In the present study, *LAMP3* is consistently gained and amplified, which supports the finding that 3q copy number gain is ubiquitous

in cervical cancer. *LAMP3*, a potential gene of interest located on 3q26, was found to be associated with metastatic potential both *in vitro* and *in vivo*.<sup>12</sup> Amplification of chromosome 3q has also been linked to lymph node metastases in two studies using comparative genomic hybridization, which exhibited an increase in 3q copy number gain in primary tumors with lymph node metastasis; however, the results by Allen et al<sup>10</sup> were not statistically significant.<sup>10,32</sup> Although the function of *LAMP3* is relatively unknown, one study suggests that *LAMP3* may be involved in tumor cell migration into surrounding lymph vessels.<sup>12</sup> *PROX1* has been found exclusively in lymphatic endothelial cells and is vital to embryonic lymphatic development.<sup>14,33,34</sup> Decreased *PROX1* expression has been found in several cancers, including hepatocellular carcinoma, biliary duct cancer, and breast cancer.<sup>35–37</sup> However, in colon cancer, overexpression of *PROX1* induces colon cancer progression by promoting a transition from a benign to a highly dysplastic phenotype.<sup>38</sup> In cervical cancer, *PROX1* lies within a region of amplification (chromosome 1q) which could suggest that *PROX1* copy number gain may function similarly to that in colon cancer. Development of cervical cancer has also been linked to a copy number gain at chromosome 5p, with *PRKAA1* being a potential gene of interest.<sup>16</sup> A recent study found that high expression of *PRKAA1* may help cancers establish their malignant nature through cellular resistance to cell death and overcoming their hypoxic condition.<sup>16,39</sup> The three genes of interest, *LAMP3*, *PROX1*, and *PRKAA1* have never been investigated in cervical cancer by using FISH. Previous studies on *LAMP3*, *PROX1*, and *PRKAA1* have focused on the level of mRNA expression by using reverse transcription-PCR and protein expression using immunohistochemistry. An advantage of multicolor FISH is the ability to visualize several genes of interest simultaneously in each cell. In addition, formalin-fixed, archived samples can be used.

Clonal patterns varied among the primary cervical cancers. To our knowledge, this is the first study investigating the difference in FISH marker patterns between the cells of a primary cervical cancer and synchronous lymph node metastases. The approach to evaluate all four gene probes and the CEP7 (Abbott Molecular, Inc) ploidy control probe within the same nuclei, allows for comprehensive pattern comparison. In our study, several cases showed the exact same clones and their frequency between primary tumor and lymph node metastasis indicating that the major clone(s) of the primary tumor were also the most successful clone(s) to establish lymph node metastasis. Therefore, in these cases, at least on the genomic level tested, the majority of cells in the primary tumor could become metastatic cells. In that case metastatic potential would be already present in the bulk of the tumor. However, in some cases, changes in the clonal constitution between the primary tumor and lymph node metastasis indicate that a pre-existing but minor clone in the primary tumor had growth advantages for establishing metastases in the lymph nodes compared with the major clone of the primary tumor. This would be consistent with the hypothesis that a subset of cells in the



primary tumor has the genetic makeup that eventually results in metastatic disease, which could indicate that additional mutations in a few cells are required for metastasis.<sup>40</sup> This difference in patterns might reflect different mechanisms in the development of lymph node metastases in cervical cancer.

In summary, we found that a composite FISH marker, comprising *LAMP3*, *PROX1*, and *PRKAA1*, allowed statistically significant discernment between patients with and without risk for metastatic disease with a reasonable sensitivity and specificity. Further studies with additional patients are warranted to validate this molecular marker prospectively in the clinical setting.

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